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KERATOCONJUNCTIVITIS OF THE KOALA, *Phascolarctos cinereus*, CAUSED BY *Chlamydia psittaci*

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Abstract: Chlamydia psittaci was cultured from 29 of 35 koalas *(Phascolarctos cinereus)* with keratoconjunctivitis. The disease progressed from acute to chronic stages over some months, with a known duration of at least 2 years. One recovered carrier was found. Up to 29% of koalas in some populations were clinically affected. A seasonal spread of infection was indicated by the high percentage of acutely affected cases found in summer. There was no evidence of susceptibility being related to age or sex.

The sera of all chronically affected koalas had complement fixation test titres against chlamydial group antigen of 80 or above; in acute cases the titre ranged from 0 to 320. Of normal koalas, 84% were serologically negative, 16% had chlamydial antibody. A titre of 80 or more considered in conjunction with ocular disease could be taken as presumptive evidence of a chlamydial etiology.

Chlamydial keratoconjunctivitis may be the ocular disease which was associated with the decline of koala numbers between the years 1885 and 1930.

INTRODUCTION

The koala, *Phascolarctos cinereus* (Goldfuss), abounded in the eucalypt forests of eastern mainland Australia a century after the arrival of the first English settlers in 1788; earlier population densities are not recorded. A marked decline in koala numbers occurred between 1885 and 1930 and was attributed to bushfires, hunting, forest clearing and disease.[10,14,17,18] Associated with this decline were epidemics of some form of ophthalmic disease,[18] but no causative agent could be identified at that time.[15]

In 1974 we reported that a keratoconjunctivitis occurred in koalas and that an agent we believed to be *Chlamydia psittaci* was isolated from affected animals.[1] In 1976 some clinical observations and response to antimicrobic therapy were recorded.[2]

This paper presents our findings on the incidence, seasonal spread and serology of chlamydial keratoconjunctivitis in koalas in New South Wales, and confirms the identity of the agent.

MATERIALS AND METHODS

Koalas and Habitat

Between 1972 and 1978, examinations were made of 105 free-living koalas near Lismore, 49 near Port Macquarie, 10 in a fauna park at Coffs Harbour, five in a Sydney fauna park and five affected koalas from four other places. Repeated observations on koalas with diseased eyes were limited as many were free-living whilst those in captivity were either mildly acutely affected and recovered after treatment[2] or were severely affected (acute or chronic) and died within three weeks of capture. The description of the disease given under Results was compiled from clinical examinations of 35 affected koalas, 25 of which were acute cases, seven were chronic and three were observed to progress from the acute stage to the chronic.

The koala habitat at Lismore and Port Macquarie consisted of isolated groups of eucalypt trees in cleared agricultural land, with some trees in urban areas utilised by koalas as well. Each area had a fairly stable community of koalas, although there was some contact between groups by nomads.[8]

Koalas were captured by forcing them from the trees then restraining them in canvas bags. Body parts were protruded from the bag as required for estimation of age,[6] determination of sex, examination of eyes and collection of blood samples. When capture was not practicable, examinations were made through binoculars; this was the case with 70% of free-living koalas.

Surveys for Incidence of Ocular Disease

To determine the incidences of keratoconjunctivitis, areas of habitat near Lismore known to be occupied by koalas were searched four times in the one year from early summer to early winter (December, 1975; February, April and June, 1976) with 196 observations on at least 85 different koalas. At Port Macquarie, similar surveys were done twice, in late summer and early winter (February and June, 1976), with 61 observations on at least 49 different koalas.

Collection of Specimens

Ocular specimens were collected by rubbing dry cotton wool swabs over the conjunctivae. Individual swabs were inserted into 3 ml of nutrient broth containing 10,000 µg/ml of streptomycin for subsequent Chlamydia culture, into 3 ml of Mycoplasma broth[9] and into Stuart's transport medium when culture for other bacteria was desired. All swabs and media were kept chilled during transport to the laboratory.

Blood samples were taken from the cephalic vein into tubes without coagulant. Serum was harvested and stored at -20 C.

A range of tissue samples (including liver, lung, spleen, kidney, peritoneal fluid, brain and retropharyngeal lymph nodes) and faeces were collected at autopsy when available. The samples were prepared for Chlamydia culture by grinding with sand and nutrient broth containing streptomycin.

Cultural Procedures

Specimens for Chlamydia culture were inoculated, usually less than 12 hours after collection, into yolk sacs of hen eggs containing five to seven day old embryos.[4] Eggs were candled daily. Embryos dead at less than five days after inoculation were discarded. Impression smears were made to detect chlamydial elementary bodies in yolk sacks of embryos dying later than five days after inoculation. These smears were stained by the modified Ziehl-Neelsen technique (MZN or semi acid fast Brucella stain[3]) or by Rivers' modification of Castaneda's method.[7] Embryos still alive at 19 days embryonic age were subcultured into fertile eggs.

For culture of other bacteria, swabs were streaked onto blood agar plates which were then cross streaked with Staphylococcus aureus to provide NAD for possible Haemophilus spp. Incubation was at 37 C for six days in an atmosphere of 5% CO_2/15%O_2/80%N_2.

The Mycoplasma broths were incubated at 37 C for five days, then streaked onto Mycoplasma agar[9] and incubated as for blood agar plates for two weeks.

Examinations of Isolates

Organisms which grew in egg yolk sacs but not on blood agar and had the morphological and tinctorial properties of chlamydial elementary bodies, were examined further according to the protocols of Page.[13]

Identification as Chlamydia: For identification of isolates as genus Chlamydia, simple boiled antigens were

prepared by the method of Dane,[5] except that the ether extraction step was omitted. Standard chlamydial group antigen prepared by the same method from *Chlamydia psittaci* isolated from a case of sporadic bovine encephalomyelitis (SBE) was obtained from the Veterinary Research Station, Glenfield, New South Wales, together with standard chlamydial antiserum from a beast convalescent after experimental infection with a similar *Chlamydia*. The test antigens were compared with the standard antigen in complement fixation (CF) tests in "chessboard" titrations of the antigens against dilutions of the standard antiserum.[7] The CF test system used 2.5 minimum haemolytic doses of complement per tube, a primary fixation time of one hour and 2% sensitised sheep erythrocytes in the haemolytic system. The optimum dilution of antigen was taken as that against which the known positive serum reacted to maximum titre. The titre of the antiserum was the reciprocal of the highest dilution giving a reading of 3 or 4 for fixation of complement with the optimum dilution of the antigen under test.

Determination of Species of Chlamydia: Sensitivity to sodium sulphadiazine was used to differentiate *C. psittaci* from *C. trachomatis;* isolates were titrated in embryonating hen eggs in the absence and presence of 1 mgm/egg of sodium sulphadiazine.[13] An isolate of *C. trachomatis* (TRIC/2/AUS/MU-9/OT) supplied by Dr. D. Graham of Melbourne University and a *Chlamydia* from a case of SBE were tested for comparison. Pathogenicity for guinea pigs, a characteristic of SBE strains,[12] was tested by intraperitoneal injection of 1.0 ml of undiluted infected yolk sac. Rectal temperatures of guinea pigs were taken daily.

Serological Procedures

Koala sera were tested for antibody in CF tests using the standard chlamydial group antigen at optimum dilution.

Statistical Analysis

Significance of survey observations was tested by the Chi-square method.

RESULTS

Clinical Description of the Disease

The earliest signs of infection were a slight serous ocular discharge, a partly or completely closed eyelid or a slight reddening of the conjunctivae, or a combination of these. During the first few days the signs increased in severity, the discharge becoming purulent and the conjunctivae reddened. Swelling of the conjunctival tissues became apparent within two weeks of the appearance of the earliest signs, leading in severe cases to eversion of the lids. The nictitating membrane became similarly swollen, sometimes to several times normal size. At any stage of the disease purulent discharge could fill the palpebral fissures, effectively blinding the animal. At about the third week, slight peripheral corneal opacity was noticeable which gradually extended centrally and became more opaque over the succeeding months.

In chronically affected koalas the conjunctival swelling was variable, pannus was observable and the corneal opacity became marked, at times complete.

All seven chronic cases were affected bilaterally; 12 of the 28 acute infections were unilateral. In four koalas the two eyes were affected to markedly different degrees.

Duration of Infection

Some information on duration of infection was gained from four animals, from each of which chlamydiae were successfully cultured.

One koala was known to be infected for 12 months, eventually dying whilst severely affected. The disease in another koala progressed in 20 months from acute early conjunctivitis to complete corneal opacity. A third koala was

observed to be severely affected 21 months after being originally discovered with a moderate infection. A fourth koala, in a fauna park in which a number of the 30 resident koalas had keratoconjunctivitis, was clinically normal when first inspected by us but was reported to have recovered from typical conjunctivitis of the left eye one year previously. Chlamydiae were isolated from ocular swabs and the CF test titre was 160. Six months later, the eyes still looked normal, ocular swabs were negative, but the titre had risen to 640.

Incidence of the Disease

In populations: At Lismore, 10 of the total of 196 observations made during the survey were positive (5%). The incidence of keratoconjunctivitis varied between communities, from zero in one group to 29% in another at the same period. At Port Macquarie, no infected koalas were found amongst the 61 observations during the survey, although three infected animals had been reported in the previous four years.

In different seasons: The incidences of clinically affected koalas near Lismore at different seasons during the survey were: early summer 2.5%, late summer 7.3%, mid-autumn 7.1%, early winter 6.5%. These variations were not statistically significant. However, of the total of 35 affected koalas from all sources which we examined over 6 years, 24 were first recorded in the summer months; 22 of these were acute infections.

According to age: The age distributions of normal and clinically affected koalas examined during the survey and of affected koalas examined by or reported to us at other times are given in Table 1. Animals older than 2 years were regarded as mature.

Of the 44 clinically affected koalas examined by or reported to us, 5% were in their 1st year, 18% in their 2nd and 77% were mature.

According to sex: Of the 39 affected koalas for which the sex was recorded, 17 were female and 22 were male.

Recovery of Chlamydiae

Chlamydiae were recovered from 29 of the 35 affected koalas examined and from the one recovered koala, but from none of 59 normal koalas with no known history of eye disease. All of the seven chronic infections yielded chlamydiae; the six unsuccessful isolation attempts were all from early cases.

Tissue and faecal specimens taken at autopsy from three infected koalas (from which chlamydiae were isolated from ocular swabs taken within 10 days prior to death) were all negative on culture for chlamydiae.

Cultures for Other Micro-organisms

The eyes of eight affected koalas in captivity from which chlamydiae were isolated were also cultured at the same time for other bacteria, including mycoplasmas, with negative results.

Identification of Chlamydiae

Growth in eggs: Chlamydiae-infected ocular material killed chick embryos either in the first passage 12 to 14 days after inoculation or in the second passage after 5 to 10 days. Dead embryos had red skins, particularly of the legs, and hyperaemic yolk sacs. Impression smears of infected yolk sacs contained elementary bodies which stained blue

TABLE 1. Ages of normal and affected koalas.

		1st Year	2nd Year	Mature
Survey	Normal	38	6	204
	Affected	0	2	8
Non-survey	Affected	2	6	26

with Castaneda's method and red with the MZN technique. No such bodies were detected in normal yolk sacs.

Identification as genus Chlamydia: The three antigens prepared from isolates from koalas from Port Macquarie, Dorrigo and Lismore gave CF test titres of 80, 80 and 160, respectively, to the known positive chlamydial antiserum. This antiserum had a titre of 80 when tested against the standard SBE chlamydial group antigen.

Determination of species: Three koala *Chlamydia* isolates had titres (egg lethal doses/ml) without and with sodium sulphadiazine of $10^{4.0}$ and $10^{4.4}$, $10^{6.1}$ and $10^{6.5}$, $10^{6.0}$ and $10^{6.0}$ respectively; the SBE agent titres were $10^{7.8}$ and $10^{8.0}$. *C. trachomatis* had $10^{3.6}$ and $10^{1.8}$ egg infective doses/ml without and with sodium sulphadiazine.

Pathogenicity for guinea pigs: Four koala *Chlamydia* isolates produced no temperature rises in guinea pigs and no abnormalities detectable on autopsy up to 42 days after inoculation. Transient chlamydial antibody titres of 5 to 40 were detected in eight of 13 guinea pigs. The SBE agent caused elevated temperatures and a fibrinous peritonitis in guinea pigs from the same colony.

Chlamydia Antibody Levels

Titres of chlamydial antibody in sera of 109 clinically normal and 28 clinically affected koalas are summarised in Table 2.

Of the clinically affected koalas 53.5% had titres of 80 or above, 14.4% were in the range of 10 to 40, and 32.1% were negative (i.e. titre < 10). All chronically affected koalas had titres of 80 or above. Acute cases had titres from 0 to 320; only acute cases had no detectable antibody.

Of the clinically normal koalas, 6.3% had titres of 80 or above (including the known recovered carrier), 9.3% were in the range of 10 to 40, and 84.4% were negative.

DISCUSSION

This investigation has shown that free-living and captive koalas suffer from chlamydial keratoconjunctivitis. The observations indicate that the disease can progress from an acute to a chronic condition of very long duration, with one or both eyes affected.

Because of the difficulty of maintaining koalas under experimental conditions, no attempt was made to satisfy Koch's postulates. The causal relationship between the *Chlamydia* and

TABLE 2. Distribution of complement fixing antibody titres from normal and affected koalas.

Titre	Clinically normal		Clinically affected				
	Number	%	Number	%	Acute unilateral	Acute bilateral	Chronic bilateral
0	92	84.4	9	32.1	5	4	0
10	4	3.7	1	3.6	0	1	0
20	3	2.8	1	3.6	1	0	0
40	3	2.8	2	7.2	1	1	0
80	2	1.8	6	21.4	3	2	1
160	4	3.6	3	10.7	0	0	3
320	0	0	6	21.4	1	2	3
640	1*	0.9	0	0	0	0	0
Totals	109	100.0	28	100.0	11	10	7

*Known recovered carrier.

the ocular signs was attested to by the isolation of the agent from 29 of 35 clinically affected koalas. This was supported by the negative results from attempted cultures of *Chlamydia* from 59 koalas with no known history of eye disease, and by negative results of cultures for other bacteria from eight clinically affected koalas.

The causative agent was culturally, morphologically and tinctorially consistent with a chlamydia. The three isolates tested antigenically were clearly *Chlamydia;* the lack of sensitivity to sodium sulphadiazine identified the species as *C. psittaci.*[13] According to Page[12] and our own work, the agent of SBE causes peritonitis in experimentally infected guinea pigs. The koala agent was not pathogenic for guinea pigs, indicating that it was not immediately derived from that of SBE. Possible relationships with avian and other mammalian chlamydiae have not been investigated.

The serological results for chronically infected koalas showed that a CF test titre of 80 or above could be taken as evidence of chlamydial infection. This is in agreement with Littlejohns[11] who wrote that, for domestic animals, "titres of 80 to 320 might be expected in animals which have recently suffered disease from chlamydial infection." Sixty percent of acutely infected koalas had titres less than 80; this suggests that insufficient time had elapsed to allow titres to develop to high levels. Because acutely infected koalas either were in captivity and satisfactorily treated for the disease[2] or were free-living, it was not practicable to rebleed the same animals at later stages to detect changes in titre. The titres detected in apparently normal koalas can be explained by prior exposure to chlamydiae either without the development of observable signs or followed by complete recovery.

The investigation also provided information on the epidemiology of the disease. The incidence varied between groups of koalas from 0 to 29%. The higher figure suggests that the disease can occur in epidemic form. The observations also suggest that spread of infection occurs more commonly in the summer months. The recovered carrier probably had a low level of multiplication of chlamydiae held in check by host defense mechnisms[16] and would have constituted an unrecognized source of infection for other koalas.

The effect of age on the occurrence of the disease should be considered in the light of the biology and population changes in koalas. The low number of second year animals (compared with first year) found in the survey is consistent with the report[8] that most of this age group disappeared each year from the Tucki Nature Reserve near Lismore; searches of surrounding areas failed to find any of them; they were presumed to have perished.

Most of the affected koalas found were mature. This would be expected as a first year koala is exposed to very little risk of infection, unless its mother is infected. The first six months of the young koala's life is spent in the mother's pouch, the second six months in close proximity to the mother. At the beginning of its second year the young koala moves further away from the parent and thus increases the chance of contacting the causative organism. As the disease is usually of long duration, a koala which became infected in its first or second year would probably grow to maturity while still infected.

Historically, it would appear likely that the eye disease associated with the koala decline from 1885 to 1930 was chlamydial keratoconjunctivitis. The previous investigations were reported[15] before cultures for chlamydiae were being undertaken in pathology laboratories. The epidemic nature of the disease would have been due to increased contact and stresses in the large populations of koalas in that period.

Acknowledgements

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